TEL-AVIV UNIVERSITY

DEPARTMENT OF BOTANY and

LABORATORY FOR ELECTRON MICROSCOPY

MEMBRANE-MEDIATED METABOLIC EFFECTS ASSOCIATED WITH THE TRANSPORT OF PHOSPHATE IN *Micrococcus Lysodeikticus*

Thesis submitted towards the MSc. degree of the Tel-Aviv University

by

Mel Rosenberg

The research work for this thesis was carried out at the Laboratory for Electron Microscopy, Tel-Aviv University, under the direction of Dr. Ilan Friedberg

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To Mom and Dad.

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INTRODUCTION

Active Transport

The main mechanism for the maintenance of the unique composition of the cell and its membrane-enclosed organelles is active transport. This term denotes those processes in which metabolic energy is expended to create and maintain certain intracellular levels of specific molecules by catalyzing their transport into or out of the cell (or organelle) against an electrochemical potential (Harold, 1972). In other words, active transport enables the accumulation of solutes which would otherwise diffuse out of the cell and the extrusion of certain molecules which would tend to diffuse into the cell. Thus active transport requires a source of metabolic energy and a sealed osmotic barrier which is provided by the biological membranes which enclose cells and organelles.

Phosphate and Metabolism

Phosphate is an essential substance in all living organisms. It is a component of vital biological molecules, such as nucleic acids and phospholipids; in addition, the phosphate ester bond plays a major role in the transfer and storage of chemical energy in the metabolizing cell.

Accumulation of Phosphate in Organisms

Phosphate may enter the cell either as orthophosphate (Pi) or as a component of a larger molecule such as a sugar phosphate (Pogell et al., 1966; Winkler,1966; Dietz and Heppel, 1971; Grover and Winkler, 1974). The catalysis of the influx and exchange of phosphate across the membranes of various cells and organelles has been extensively investigated.

In yeast (Bartlett, 1958) and red blood cells (Borst-Powvells et al., 1962), Pi is esterified upon entry into the cell. In sea urchin egg cells, fertilization is accompanied by a dramatic increase in the rate of Pi uptake; the uptake is almost unaffected by various inhibitors of metabolic energy (Chambers and Whiteley, 1966). Phosphate-deprived Euglena acquire the ability to rapidly accumulate Pi (Blum, 1966).

A number of general properties of phosphate transport may be gleaned from the various investigations. Pi permeates intact biological membranes to a significant extent only in the presence of specific carriers (Mitchell, 1953,1954). The ability of cells to take up phosphate often develops in response to phosphate deprivation or physiological changes. Analogs of phosphate, such as arsenate, can usually permeate through the same systems as phosphate. The translocation of phosphate is invariably inhibited by sulfhydryl reagents, such as N-ethylmaleimide, and is usually curtailed by various inhibitors of metabolic energy production and transfer. Certain cations generally have a stimulatory effect on Pi uptake (an exception is the Euglena, in which K^+, Li^+ and Na^+ inhibit Pi transport in the presence of Tris buffer (Blum, 1966)). Many microorganisms

accumulate phosphate to high levels, storing most of it as inorganic polyphosphate (Harold, 1966).

Transport of Phosphate in the Mitochondria

Mitochondria possess at least one specific mechanism for the translocation of phosphate across the mitochondrial membrane (Tyler, 1969). The transport is non-electrogenic; no net charge is translocated in the process. Stoichiometric calculations and results obtained with the use of ionophores have indicated that each phosphate molecule enters the mitochondria accompanied by the influx of almost two protons (Palmieri et al., 1970). K^+ stimulates phosphate transport, presumably by exchanging for protons (Meisner et al., 1972). Phosphate can also enter the mitochondrion on the dicarboxylic acid porter, exchanging either another Pi molecule, or one of malate or succinate (Hoek et al.,1971).

The general view that two distinct systems thus exist for Pi transport has been recently challenged by Lofrumento et al. (1974).

Even under conditions which inhibit energy metabolism and transfer (at 0° C, or in the presence of oligomycin and cyanide), net uptake of Pi against its concentration gradient does occur (Coty and Pederson, 1974).

Transport of Phosphate in Bacteria

Transport of phosphate has been studied in various bacteria.

M. pyogenes- In his pioneering work, Peter Mitchell showed the presence of an osmotic barrier for Pi transfer at the cell surface, and the presence of a specific system for Pi uptake (Mitchell, 1953, 1954). This system is inhibited by sulfhydryl reagents, certain anions, and uncouplers of oxidative phosphorylation. Under the conditions studied by Mitchell (the cells were not previously starved for phosphate) net uptake against a concentration gradient was not reported.

E. coli- Pi transport in E. coli has been extensively studied by Medveczky and Rosenberg (1970,1971). They found that rapid Pi uptake is observed only in cells which have been starved for phosphate.

The uptake of Pi in starved cells is energy-dependent and has two phases: a primary phase of about two minutes duration, characterized by a high affinity for Pi and a rapid rate of uptake, followed by a second phase which exhibits lower affinity for Pi and a slower transport rate (Bennett and Malamy, 1970; Rosenberg and Medveczky, 1971). Part of the phosphate accumulated during the initial phase can exchange when high external concentrations of Pi or arsenate are added. The initial rate of efflux upon exchange is the same as the initial rate of Pi influx.

A phosphate-binding protein, which is involved in the Pi transport system has been isolated and purified (Medveczky and Rosenberg, 1970). Upon osmotic shock, the binding protein is released from the periplasmic space and the Pi transport rate is subsequently reduced. Pi uptake can be partially restored to shocked cells and to mutants defective in the binding protein by preincubating these cells with binding protein. This protein has recently been shown to be identical to the product of an alkaline phosphatase regulatory gene, R2a (Gerdes and Rosenberg, 1974;

Garen and Otsuji, 1964).

Various mutants of Pi transport in E. coli have been isolated by selection for resistance to arsenate (Rosenberg and Medveczky, 1971). One class of mutants exhibited low phosphate uptake and no biphasic uptake as in the wild type. The second class of mutants was deficient in binding protein. One exceptional mutant took up Pi to a much greater extent than the parent cells, with no break in the transport rate.

Weiden et al. (1967) studied the potassium requirement for Pi transport in E. coli. When cells are starved for K^+, their Pi uptake is low, whether the growth medium contains high Pi concentrations or low Pi levels. Upon addition of K^+, potassium and phosphate are both rapidly taken up by the cells, concurrent to a substantial increase in respiration rate.

Bacillus Cereus- The cells accumulate phosphate by an energy-dependent process (Rosenberg and La Nauze, 1968; Rosenberg et al., 1969). Rate of uptake is doubled in cells which have been grown without phosphate for two or more hours.

Pi which enters the cell fills a primary phosphate pool, which can also be filled by arsenate and pyrophosphate, both of which share the Pi transport system. When the primary pool is filled, the rate of uptake is halved, and esterification of Pi is observed. The kinetics of Pi uptake by B. cereus resemble those of E. coli.

The initial rate of Pi uptake shows little variation when K^+, Na^+, or triethanolamine are added as counter ions. The subsequent secondary uptake is stimulated by K^+ (at pH=7.2). Glucose and succinate can serve as energy donors; inhibition is obtained with azide and 2,4 dinitrophenol, indicating that respiration and proton gradient are involved in the transport.

Streptococcus faecalis- Uptake of Pi in S. faecalis is dependent on metabolic energy; glycolysis supports rapid uptake under anaerobic conditions (Harold et al., 1965). Pi is taken up for about an hour, after which time a constant internal level of ^{32}Pi is maintained. While uptake is stimulated by K^+, valinomycin in the presence of K^+ does not inhibit uptake although proton ionophores do.

Streptococcus strain SL-1- Isolated and investigated by Tanzer et al. (1969), this strain accumulates Pi in the presence of glucose. The transport is essentially unidirectional and is coupled to net proton efflux.

Corynebacterium bovis- The initial rate of Pi uptake by Pi-limited cells was found to be dependent on the internal Pi concentration as well as the external (Chen, 1974). Both the initial transport rate and the transport affinity for Pi decreased with increasing internal concentrations of Pi.

Micrococcus lysodeikticus- Pi transport by M. lysodeikticus is currently being investigated in this laboratory (Friedberg, 1975). It was found that cells which have been starved for phosphate carry out rapid (more than 150 nmoles Pi/min/mg cell protein under optimal conditions) uptake of phosphate by a specific, carrier-mediated process, against a concentration gradient. Transport is energy-dependent, but no exogenous energy source need by supplied for rapid uptake. The rate of uptake in cells which have not been starved for phosphate is one or two percent of that in starved cells.

In contrast to the kinetics of Pi uptake observed in E. coli and B. cereus, the uptake in M. lysodeikticus is linear for the initial twenty minutes.

Pi transport is stimulated by up to 2mM Mg^{++}, and by K^{+} concentrations of up to 100mM. Arsenate is a competitive inhibitor. The transport is inhibited by sulfhydryl reagents, uncouplers of oxidative phosphorylation, and partially blocked by inhibitors of respiration. The apparent Km for Pi influx was found to be 4.3uM.

Orthophosphate taken up by the cells is rapidly incorporated into a trichloroacetic acid-insoluble fraction, composed mainly of polyphosphate (Esh'har, 1968; Friedberg and Avigad, 1968).

The rates of transport and the ensuing metabolism of Pi in phosphate-starved cells of M. lysodeikticus reaches much higher levels than in other bacteria studied to date; therefore the uptake of phosphate in this microorganism seemed a promising subject for investigation.

Characteristics of the Cytoplasmic Membrane of M. lysodeikticus

The general composition of the cytoplasmic membrane of M. lysodeikticus has been studied (Gilby et al., 1958; Salton and Freer, 1965).

Enzymatic activities of membrane and cytoplasmic fractions of M. lysodeikticus as determined by Mitchell (1963) are presented in Table I. It can be seen that lactic, succinic, and α-ketoglutaric dehydrogenase activity, as well as NADH oxidase activity is found almost exclusively in the membrane fraction. On the other hand, alcohol and isocitric dehydrogenase activity is concentrated in the cytoplasm. ATPase activity was found to be distributed between the membrane and cytoplasm. More recently, the membrane-bound ATPase has been isolated, purified, and well characterized (Schor et al., 1974).

Respiration rates of isolated membranes on a variety of energy substrates is given in Table II (Grinius et al., 1972). It was found that while intact protoplasts respire on succinate much the same as intact cells, upon lysis the rate of succinate oxidation is radically reduced (Shah and King, 1966), whereas Ellar et al. (1971) have more recently shown that succinic dehydrogenase activity of a membrane fraction could be restored by preincubation with succinate.

Isolated membranes of M. lysodeikticus are not capable of efficiently coupling phosphorylation to respiration, as are other bacterial membrane preparations (Jones et al., 1971; H.R. Kaback, personal communication). Ishikawa and Lehninger (1962) obtained a maximal P:O

ENZYME	ACTIVITY nmole substrate/ g/sec	
	Membrane	Cytoplasm
Cytochrome oxidase	117	14.8
Alcohol dehydrogenase	40	143
NADH oxidase	500	49
Lactic dehydrogenase	44	0.3
Succinic dehydrogenase	44.1	2.1
α -ketoglutarate dehydrogenase	5.6	1.64
Isocitric dehydrogenase	9.9	84.2
ATPase (35°)	3.24	4.28

TABLE I - Enzymatic activity of Membrane and Cytoplasmic Fractions of M. lysodeikticus. All assays were carried out at 25° except where otherwise noted (Mitchell, 1963).

SUBSTRATE	RESPIRATION RATE nmoles O/min/mg protein	PHOSPHORYLATION RATE nmoles ATP/min/mg protein	P:O RATIO
Malate	330	39	0.08
Lactate	240	32	0.13
Succinate	90	25	0.28
NADH	200	not determined	-
Ascorbate-TMPD	10	0	0

TABLE II - Respiration and Phosphorylation of sonicated membranes of M. lysodeikticus. From the results of Grinius et al. (1972)

ratio of 0.5 for phosphorylation during respiration on NADH. Grinius et al. (1972) also obtained low phosphorylation values (Table II).

Grinius et al. (1972) found that oxidation of NADH, succinate, malate and lactate in sonicated particles of M. lysodeikticus is coupled to anion uptake. This indicates an inside-positive electric potential across the membrane, while the polarity is inside-negative in whole cells. This may be explained by the observation that sonication turns a sizeable portion of the membrane vesicles inside-out with respect to their in vivo orientation (Gorneva and Ryabova, 1974; Altendorf,1974; Futai, 1974). Generation of a membrane potential in these sonicated particles is prevented and abolished by cyanide and uncouplers, and cannot be energized by external ATP.

K^+ accumulation in the presence of valinomycin was observed during the oxidation of malate, lactate, and ascorbate-TMPD by right-side out vesicles (Gorneva and Ryabova, 1974). While the authors postulate that this is an expression of interior-negative electric potential coupled to substrate oxidation, it was not shown that the K^+ flux is not at least partly due to permeation of the electron donor anions into the vesicles.

Transport in Bacterial Membrane Vesicles

Bacterial membrane vesicles, prepared according to methods developed by Kaback (1971), have been shown capable of energy-coupled uptake of a wide variety of metabolites. Uptake of amino acids, sugars and other substrates against their respective concentration gradients has been demonstrated in vesicles isolated from a number of bacteria.

Energy is usually supplied by natural and artificial electron donors, but may also be provided by induction of a membrane potential by other means (for example, by preloading the vesicles with K^+ and then adding valinomycin) (Kashket and Wilson, 1973; Hirata et al., 1973). Bacterial membrane vesicles have not been able to couple phosphate bond energy to transport, though the high energy phosphate bond can energize such transport in intact cells (Klein and Boyer, 1972; Berger, 1973).

The mechanisms responsible for coupling electron flow in membrane vesicles to active transport has been a matter of dispute in recent years (Lombardi et al., 1974; Harold, 1974). While it is clear that in intact cells, a major role in the energy coupling for active transport is played by the extablishment of a proton gradient across the membrane (West and Mitchell, 1973; Essenberg and Kornberg,1975), a symport of substrate and protons in vesicles has yet to be demonstrated (H.R. Kaback, personal communication).

Purpose of this Study

The purpose of this work was to gain information about the active transport mechanism for phosphate on the molecular level by studying a number of membrane-mediated changes in metabolism concurrent to Pi transport in M. lysodeikticus.

Among the effects studied were changes in the rate of respiration, cation fluxes across the membrane, and changes in the carotenoid spectrum.

Experiments were carried out on intact bacteria and isolated membrane vesicles.

MATERIALS AND METHODS

Materials

All the materials used in the experiments were of analytical grade.

Bacterial Strain

M. lysodeikticus, ATCC 4698 (Fleming) was used throughout.

Growth Media

1) Slants- The slants contained 0.8% Difco nutrient broth and 2% Difco bactoagar, brought to pH 7.8 with KOH.

2) YC-Medium- A rich medium, usually used for starter, was composed of 0.1% yeast extract (Difco), 0.5% casamino acids (Difco, sterilyzed in autoclave), and salt solutions (sterilyzed by filtration through Millipore filters, 0.45 um pore size) composed of 4.2 g/l $NaHCO_3$, 0.4 g/l $MgSO_4 \cdot 7H_2O$, 20 mg/ml $FeSO_4 \cdot 7H_2O$, 16 mg/ml $MnSO_4$ (Esh'har, 1968).

3) P-AVS medium- A defined medium, containing phosphate, amino acids, vitamins, and salts in the following concentrations: 5 g/l L-glutamic acid, 1 g/l L-arginine·HCl, 0.5 g/l L-tyrosine, 0.4 g/l L-phenylalanine, 0.02 g/l D,L-methionine; 1.36 g/l KH_2PO_4 (final concentration 10 mM), 5 g/l NaCl, 1 g/l NH_4Cl, 0.02 g/l $MgSO_4 \cdot 7H_2O$, 0.005 g/l $FeSO_4 \cdot 7H_2O$; 60 mg/l Inosine, 2 mg/l Thiamine, 1 mg/ml Biotin.

   The vitamins and the $FeSO_4 \cdot 7H_2O$ solutions were each filtered separately through Millipore filters, 0.45 um pore size. The solution of amino acids and the other salts was brought to pH 7.8 with KOH and autoclaved (Esh'har, 1968).

4) TEA-AVS medium- A defined medium without phosphate, this medium was composed the same as P-AVS medium, except the KH_2PO_4 was replaced by 9.29 g/l triethanolamine hydrochloride , to obtain a final concentration of 50 mM (Friedberg, 1975).

Growth Procedure

Bacteria were maintained on slants at 4°. Cells from these slants were innoculated into YC-medium and grown at 30° in a shaker. Each hundred ml of P-AVS medium was then innoculated with 1-3 ml of cells grown in YC-medium and grown at 30° in a shaker until middle to late log-phase. The cells were harvested by centrifugation (10,000 g, 10 min, room temperature) and washed twice in 100 ml of TEA-AVS medium, minus the $FeSO_4 \cdot 7H_2O$ and vitamins. These washed cells were then innoculated into Pi-starvation medium (TEA-AVS) to a turbidity of about 100 Klett Units (KU_{55}), and grown for about nine hours at 30° in a shaker. The cells were harvested in late log-phase, after the turbidity of the growth medium had at least doubled. These cells (denoted Pi-starved cells)

were harvested and washed twice in buffer containing 50 mM triethanolamine·HCl, pH 7.8, and KCl and $MgCl_2$ (or $MgSO_4 \cdot 7H_2O$) in those concentrations used in the subsequent experiment. The cells were then resuspended in the same buffer.

Membrane Preparation

Procedure I- Pi-starved cells were suspended 1:50 (one gram wet cells in 50 ml buffer) in 50 mM triethanolamine·HCl, pH 7.8, containing 100 mM KCl, 5 mM $MgSO_4$, and 1 M sucrose. The bacterial suspension was then aerated in a shaker at 37° for twenty minutes to facilitate lysis. Lysozyme was added to a final concentration of 60 ug/ml (in the ratio of one gram lysozyme to 500 g wet cells). Lysis was followed by the decrease in turbidity of diluted samples. Within 30 minutes lysis was complete. The lysate was then dialyzed at room temperature against four volumes of the same buffer, minus sucrose. The concentration of sucrose which diffused into the external buffer was measured with a Bausch and Lomb refractometer. It was found that the sucrose level in the external buffer rose to 0.15 M within two hours. At this time the lysate was centrifuged (40,000 g, 20 min, room temperature). The supernatant was discarded and the pellet (containing the membranes) was gently resuspended in the same buffer, containing also 0.2 M sucrose, to a final concentration of 5 - 10 mg protein/ml.

The membrane preparations were either used immediately or were frozen in liquid nitrogen and stored at -20°.

Procedure II- The procedure was similar to Procedure I, but with the following changes:

1) Dialysis proceeded overnight at 8°.
2) 5 mM DTT and 0.5 mM ATP were added prior to lysis and were present in the same concentration in the dialyzing buffer. The membranes were resuspended after centrifugation in buffer also containing 0.2 M sucrose and 5 mM DTT, but with no ATP.

Procedure III- The procedure was similar to Procedure I, with the following change: after lysis in the buffer described in Procedure I, the lysate was centrifuged (40,000 g, 60 min, room temperature). The pellet was resuspended to 5 - 10 mg protein/ml in buffer and used immediately in oxygen consumption assays. Sucrose was omitted from this procedure.

Uptake Assays of Pi and Proline in Intact Cells

Cells were harvested by centrifugation, washed twice with 50 mM triethanolamine·HCl buffer, pH 7.8, containing concentrations of KCl and $MgSO_4 \cdot 7H_2O$ as specified, and resuspended in the same buffer 1:50. For the standard uptake experiment, cells were suspended in the buffer (200 ul of 1:50 cell suspension per 1 ml), in flat-bottom flasks, to ensure adequate aeration. Measurements of uptake were initiated by addition of 100 uM ^{32}Pi or 10 uM ^{14}C-proline. Samples were withdrawn after 15, 30, 60, 120 s (unless otherwise indicated), filtered through a membrane filter of 0.45 um pore size (Sartorius), and immediately washed

with 5 ml of buffer. The filters were dried, and the radioactivity of the material on the filters was measured with a Packard 3330 scintillation spectrometer. The amount of radioactivity was plotted vs. time and the uptake rate obtained from the slope.

Uptake of Pi and Proline by Isolated Membranes

The standard assay system (final volume = 1ml) contained 50 mM triethanolamine·HCl, pH 7.8, 100 mM KCl, 5 mM $MgSO_4 \cdot 7H_2O$, 0.2 M sucrose, membranes to a concentration of 1-2 mg protein/ml, 20 mM energy source, and other additions as specified.*

Uptake was initiated by the addition of 100 uM ^{32}Pi or ^{14}C-proline. Samples of 200 ul (unless otherwise indicated) were taken at various time intervals, filtered through a membrane filter of 0.45 um pore size (Sartorius), and immediately washed with 50 mM triethanolamine·HCl, pH 7.8, containing 100 mM KCl, 5 mM $MgSO_4 \cdot 7H_2O$, 0.2 M sucrose and 0.5 M LiCl (to prevent radioactivity from escaping from the vesicles during washing). The filters were dried, and the radioactivity was measured as in uptake assays with intact cells.

Oxygen Consumption Assay

Oxygen consumption was measured with a Clark-type oxygen electrode (Beechey and Ribbons, 1972). Temperature was maintained at 30°. Oxygen concentrations were calibrated according to the Handbook of Chemistry and Physics, The Chemical Rubber Co, Cleveland, 1951-52.

A standard assay contained 0.5-1 mg cell protein/ml, 50 mM triethanolamine·HCl, pH 7.8, 10 mM KCl, 5 mM $MgCl_2$, and other additions as indicated, to a final volume of 5 ml.

Proton Flux Assay

Proton flux was measured with a glass pH electrode. The calomel reference cathode was suspended in a solution of 1M KCl and was connected to the cell suspension by a salt bridge containing 1 M KCl in 2% agar. Changes in hydrogen ion concentration of the cell suspension were calibrated by the addition of known concentrations of HCl. Temperature was maintained at 30°. Final assay volume was 3 ml. Pi-starved cells were suspended to a concentration of 1.8 mg cell protein/ml in 1 mM triethanolamine.HCl, pH 7.2, 1 mM KCl, and 2 mM $MgCl_2$. Phosphate was added as the triethanolammonium salt (TEA-Pi).

* 5 mM DTT was added as well in assays with membranes isolated according to Procedure II.

Potassium Flux Assay

Potassium flux was measured with a Beckman cationic electrode (cat. no. 39137). The calomel reference electrode was suspended in 1 M triethanolamine·HCl, brought to pH 7.8 with NaOH, and connected to the cell suspension by a bridge containing 1 M triethanolamine·HCl, pH 7.8, in 2% agar. Pi-starved cells (1.4 mg cell protein/ml) were suspended in 50 mM triethanolamine·HCl, brought to pH 7.8 with NaOH, 1 mM KCl and 2 mM $MgCl_2$. Changes in potassium ion concentration were calibrated with known concentrations of KCl. Pi was added as the triethanolammonium salt.

Protein Determination

Protein was determined by the Biuret reaction (Gornell *et al.*, 1949). The Biuret reagent was composed of 0.15% $CuSO_4.5H_2O$, 0.6% sodium potassium tartarate in 3% NaOH.

To 0.5-2 mg protein in 0.6 ml, 2.4 ml of Biuret reagent were added, and after 30 min incubation the colour intensity was read in the Uvispeck spectrophotometer (Hilger & Watts) at 550 nm.

Whole cells were lysed prior to protein determination by treatment with lysozyme. Lysozyme, 200 ug/ml, and DNAse, 1 ug/ml were added to bacterial suspensions of 2-8 mg dry wt/ml; the suspension was incubated for 20 min at 37° to accomplish cell lysis.

The following modified procedure was used to accomplish lysis and improve dissolution of the membrane fraction which otherwise causes turbidity which interferes with the colorimetric determination: samples of 1.8 ml containing 4.5% NaOH were simmered at 100° for 10 min and then cooled; 1.2 ml of a solution containing 0.3% $CuSO_4.5H_2O$ and 1.2% tartarate was added, and the assay was completed as described above.

RESULTS

Effect of Pi on Rate of Oxygen Consumption in Intact Cells

The uptake of Pi by Pi-starved cells of M. lysodeikticus is an energy-dependent process, and is greatly inhibited by respiratory inhibitors (Friedberg, 1975). Since respiration is the main energy source in such aerobic bacteria, it was of interest to see whether the addition of Pi to Pi-starved cells would have an effect on the respiration rate.

Pi-starved cells, suspended in triethanolamine·HCl buffer, pH 7.8, at 30° were found to consume oxygen at the rate of 7.5 ± 2.5 nmoles O_2 per minute per mg cell protein (Table III). These variations in the rate of endogenous respiration in different experiments might be affected by the physiological state of the bacteria due to their growth, time of incubation in buffer prior to the experiment, duration of Pi starvation, and so on. Cells grown on defined medium (P-AVS) with phosphate respired endogenously at about the same rate as starved cells (Table IV).

Addition of external energy sources, such as succinate and ethanol resulted in a marked increase in respiration rate of both Pi-starved (Tables III,V,VI; Figs. 1,2,3) and non-starved (Table IV) cells. Optimal stimulation of respiration with ethanol was obtained at a concentration of 2% (Table III, expt. 5). Respiration was inhibited almost completely by low concentrations of cyanide (Table III, expt. 1; Table IV).

The respiration of Pi-starved cells was found to increase greatly upon addition of Pi (Tables III,V,VI; Figs. 1,2,4,5) and arsenate (Table III, expt. 1,5; Fig. 3). This phenomenon was specific for Pi (and its analogue, arsenate) added to Pi-starved cells. The respiration of cells grown in the presence of Pi was not significantly affected by addition of Pi or arsenate (Table IV). The addition of 10 mM concentrations of KCl, $MgCl_2$, and NaCl each had little or no effect on the respiration rate (data not presented), indicating that the respiratory increase by Pi is not a general salt effect. Proline, which is transported into M. lysodeikticus (see Results, Proline Uptake) affected neither the res-

piration of Pi-starved (Table III, expt. 2) nor non-starved (Table IV) cells. This shows that the effect is not general for transported metabolites.

When Pi is added to Pi-starved cells after addition of ethanol or succinate, an additional increase in respiration occurs simultaneously (Tables III,V,VI; Fig. 2). Similarly, succinate, added after the addition of Pi or arsenate also further increases the respiration rate in Pi-starved cells (Table III; Fig. 1). Although it seems from these results that the stimulation of respiration by electron donors and Pi are independent, a certain degree of dependence was indeed shown (see below).

Experiments were performed to follow the kinetics of the Pi-dependent respiration. Low Pi concentrations of upto 80 uM stimulated the respiration for a short period followed by a return to a lower level (Table V; Fig. 4); this is evidently due to disappearance of inorganic phosphate from the system as it is rapidly polymerized inside the cells (see Results, P:O Ratio). Addition of higher Pi concentrations (above 120 uM) resulted in an initial linear increase in respiration which was followed by a further linear increment (Table V; Fig.5).

When the effect of Pi concentration on the initial stimulation of the respiratory rate was examined, typical saturation kinetics were found. When the data were plotted according to Lineweaver-Burke (Table V; Fig.6) a constant of 85 uM was obtained for half-saturation of the stimulation in respiration; the same result was obtained also for addition of various Pi concentrations to cells respiring on 1% ethanol (Table VI; Fig. 7). On the other hand, V_{max} values for respiration were 30 nmoles O_2/min/mg protein in the presence of ethanol, and 15.6 in the absence thereof. It was also found that the increase in respiration rate by a given Pi concentration was greater in the presence of ethanol (compare Table V (a) with (g) and (d) with (h)). The observation that the extent of stimulation by Pi is increased in the presence of ethanol indicates that the respiratory increase upon Pi addition to Pi-starved cells is at least partially dependent on an energy source.

Table III- Effect of various additions on the respiration rate of Pi-starved cells.

Expt.	Assay	Addition	Rate of O_2 Consumption (nmoles O_2/min/mg protein)	Relative Rate	Remarks
I	a	-	9.3	1.00	
		Pi, 4 mM	27.0	2.90	
		KCN, 1 mM	0.0	0.00	
	b	-	6.7	1.00	
		KH_2AsO_4, 4 mM	22.4	3.34	Initial rate
			12.0	1.79	Seven min after arsenate added; See Fig. 3
II	a	-	9.0	1.00	
		Pi, 4 mM	29.4	3.27	See Fig. 1
		Succinate, 10 mM	57.7	6.41	
		KCN, 0.4 mM	3.6	0.40	
	b	-	6.6	1.00	
		Succinate, 10 mM	29.4	4.90	
		Pi, 4 mM	55.3	9.22	See Fig. 2
	c	-	8.4	1.00	
		Succinate, 10 mM	30.7	3.65	
		Proline, 5 mM	27.6	3.29	
		Pi, 4 mM	51.2	6.10	
III	a	-	5.2	1.00	
		Pi, 5 uM	5.6	1.08	
		Pi, 200 uM	14.9	2.86	
		Pi, 200 uM	21.5	4.13	
		Pi, 200 uM	24.2	4.65	
		Pi, 200 uM	24.2	4.65	
	b	-	4.8	1.00	
		Pi, 100 uM	12.5	2.60	
		Pi, 100 uM	19.4	4.04	
		Pi, 100 uM	23.4	4.87	
		Pi, 100 uM	27.4	5.71	
	c	-	6.1	1.00	
		EtOH, 1%	20.5	3.36	
		Pi, 4 uM	33.0	5.41	
	d	-	6.0	1.00	
		EtOH, 1%	19.7	3.28	
		Pi, 25 uM	36.4	6.07	

Table III cont.

Expt.	Assay	Addition	Rate of O_2 Consumption nmoles O_2/min/mg protein	Relative Rate	Remarks
IV		-	8.0	1.00	
		EtOH, 1%	22.0	2.75	
		Pi, 10 mM	64.0	8.00	
V	a	-	10.0	1.00	
		Pi, 100 uM	28.1	2.81	
		Succinate, 10 mM	40.8	4.08	
	b	-	10.0	1.00	
		KH_2AsO_4, 100 uM	36.1	3.61	Initial rate
			28.1	2.81	Five minutes after arsenate added
		Succinate, 10 mM	35.0	3.50	Succinate added 5 min after arsenate; respiration on succinate linear thereafter
	c	-	9.0	1.00	
		EtOH, 0.5%	19.5	2.17	
		EtOH, 0.5%	22.0	2.44	
		EtOH, 1%	23.5	2.61	
		EtOH, 1%	18.0	2.00	
		Pi, 100 uM	41.6	4.62	

Table III- Effect of various additions on the respiration rate of Pi-starved cells

Pi-starved bacteria were washed, resuspended and assayed for oxygen consumption in 50 mM triethanolamine·HCl buffer, pH 7.8, 10 mM KCl and 5 mM $MgCl_2$, as described in Methods. Endogenous respiration and respiration with various subsequent additions was linear with time unless otherwise indicated. Presented in the table are the rates of endogenous O_2 consumption, followed by respiration rate after each subsequent addition. Each experiment was performed with a different batch of bacteria; the miniscule letters denote different assays in the same experiment. The relative rate, which is calculated relative to the endogenous respiration rate for the particular assay, is also given.

Table IV - Effect of various additions on the respiration rate of cells not starved for Pi.

Assay	Addition	Rate of O_2 Consumption (nmoles O_2/min/mg protein)	Relative Rate
a	-	7.0	1.00
	Pi, 4 mM	6.7	0.96
b	-	7.4	1.00
	Proline, 5 mM	7.4	1.00
c	-	6.2	1.00
	Succinate, 10 mM	40.0	6.45
	KCN, 1.6 mM	0.8	0.13
d	-	8.8	1.00
	EtOH, 1.6%	39.3	4.46
e	-	5.5	1.00
	KH_2AsO_4, 1 mM	5.5	1.00
	Succinate, 10 mM	35.5	6.36

Table IV- Bacteria were grown on P-AVS medium, washed, resuspended and assayed for oxygen consumption in 50 mM triethanolamine·HCl buffer, pH 7.8, 10 mM KCl and 5 mM $MgCl_2$. Oxygen consumption was assayed as described in Methods. The results presented are from a single experiment. Subsequent additions were added five minutes after previous additions; all the rates were constant with time.

Table V - Effect of various Pi concentrations on the respiration rate of Pi-starved cells.

Assay	Respiration Rate Prior to Pi Addition (nmoles O_2/min/mg protein)	Pi conc. added (mM)	Initial Rate of Respiration after Pi added (nmoles O_2/min/mg prot.)	Duration of Initial Rate of Respiration (min)	Secondary Rate of Respiration after Pi added (nmoles O_2/min/mg prot.)
a	5.7	0.04	9.9	2.5	0.0
b	6.6	0.08	13.8	4.5	5.5
c	3.6	0.12	14.7	3.0	16.5
d	4.7	0.20	15.2	2.5	17.1
e *	4.7	0.40	17.1	2.5	22.0
f	6.4	4.00	20.7	2.5	22.5
g *	26.1	0.04	42.0	1.0	27.7
h	25.9	0.20	46.1	1.0	55.0

Table V- Oxygen consumption of a suspension of Pi-starved bacteria (1.5 mg protein/ml) in 50 mM triethanolamine·HCl, pH 7.8, 10 mM KCl, 5 mM $MgCl_2$ was measured as described in Methods. 1% EtOH was present in assays g and h; thus the respiration rates in these assays are given in the presence of ethanol. The respiration rates in the first six assays were measured in the absence of exogenous energy donors and the initial rates of respiration after Pi addition minus the rates prior to Pi addition are used in the plots of Fig. 6.

* Traces of these assays are presented in Figs. 5 and 4, respectively.

Table VI - Effect of various Pi concentrations on the respiration rate of Pi-starved cells.

Assay	Respiration Rate * (nmoles O_2/min /mg protein	Pi conc. added (uM)	Respiration Rate after Pi Addition (nmoles O_2/min /mg protein)	Increase in Rate of Respiration (nmoles O_2/min /mg protein)
a	18.7	-	-	-
b	16.4	4	17.9	1.5
c	15.4	8	17.6	2.2
d	14.6	16	19.4	4.8
e	15.0	24	22.0	7.0
f	14.3	32	23.0	8.7
g	15.0	40	24.9	9.9

Table VI - Oxygen consumption of Pi-starved cells, suspended to a concentration of 3.7 mg protein/ml in 50 mM triethanolamine·HCl, pH 7.8, 10 mM KCl, and 5 mM $MgCl_2$, was assayed as described in Methods. Ethanol was added to a final concentration of 1% two minutes prior to Pi addition. The rate of oxygen consumption in the presence of various Pi concentrations was measured from ten seconds after Pi addition, when a linear response was obtained. The increase in the rate of respiration was used in the plots of Fig. 7.

* in the presence of 1% EtOH, prior to Pi addition

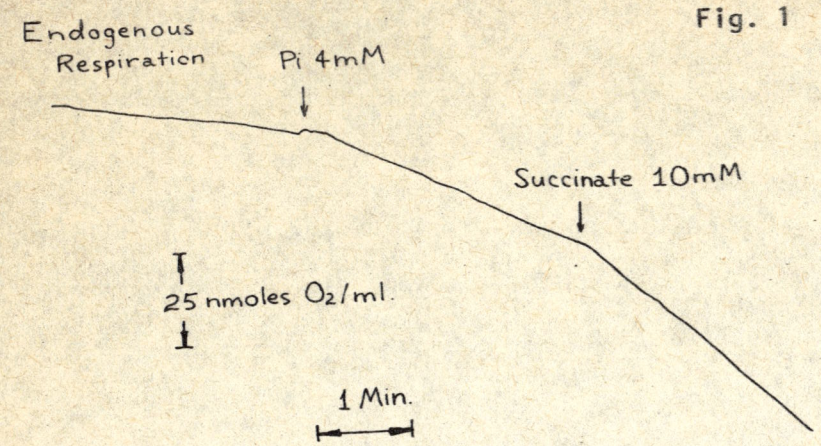

Fig. 1

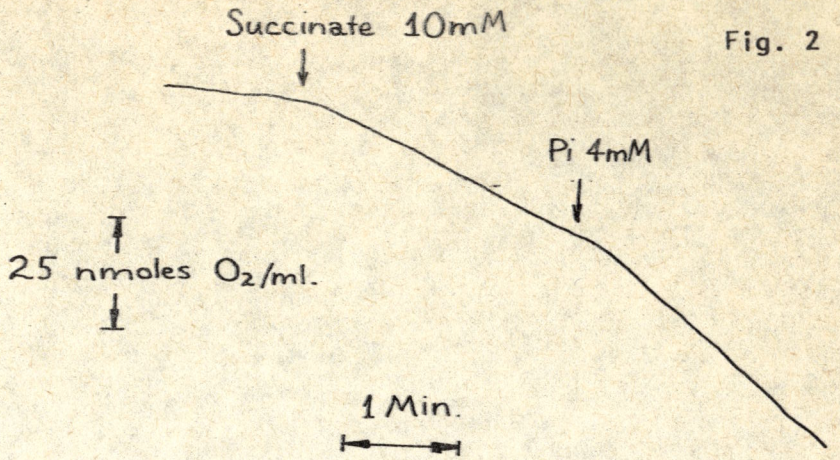

Fig. 2

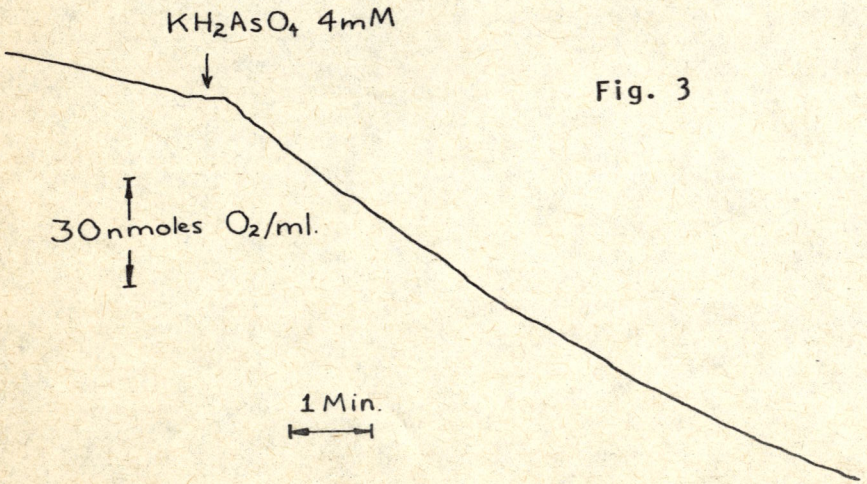

Fig. 3

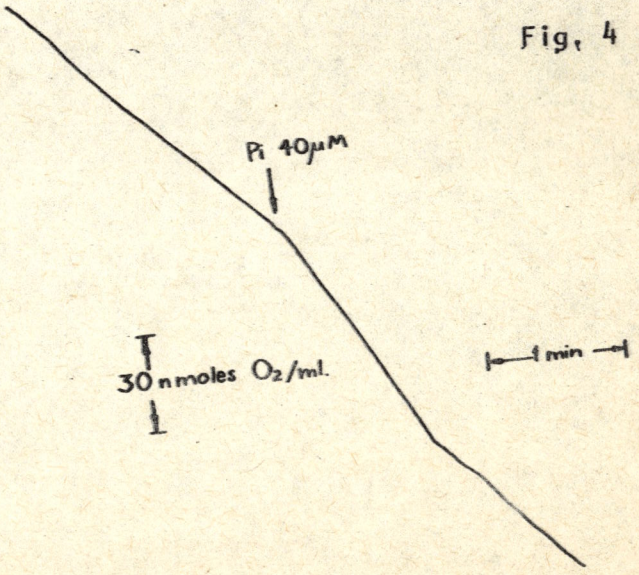

Fig. 4

Figures 1-5 - Traces of Respiration of Pi-starved cells.
Pi-starved cells were assayed for oxygen consumption as described in Methods and in Tables III and IV. Additions are indicated on the traces.

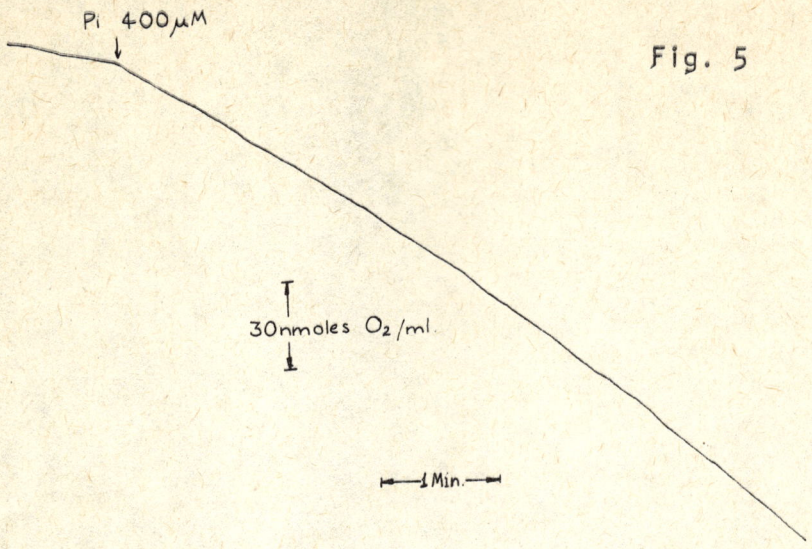

Fig. 5

Fig. 5

Fig. 6

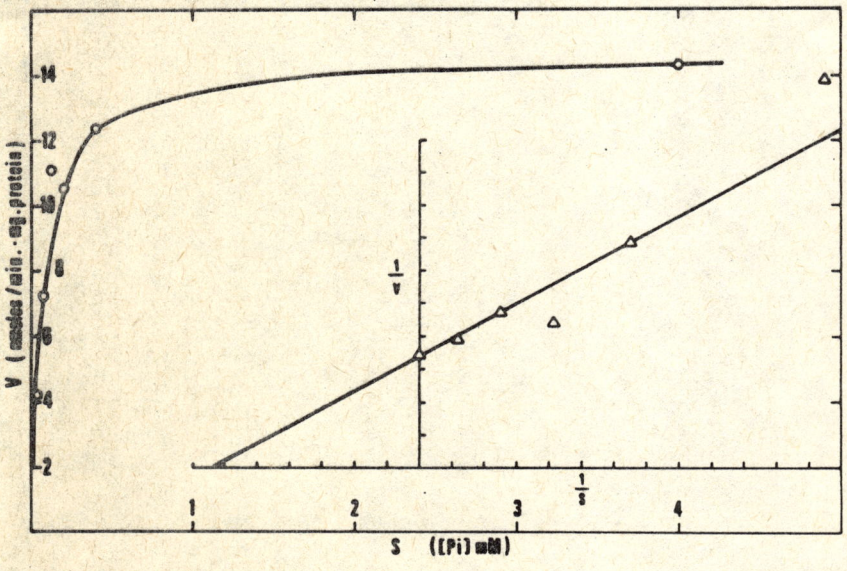

Fig. 7

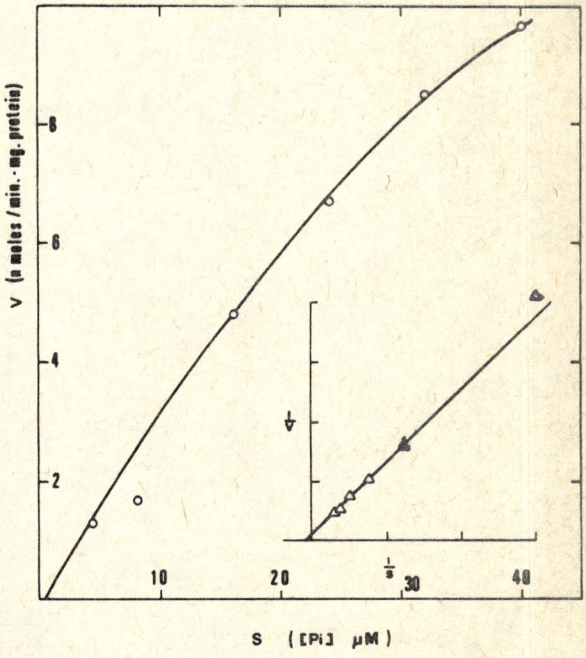

Fig. 6 - Increase in the initial rate of respiration (V) as a function of Pi concentration (S), in the absence of exogenous electron donor. Inserted is a Lineweaver-Burke plot of 1/V as a function of 1/S.

Fig. 7 - Increase in the initial rate of respiration (V) as a function of Pi concentration (S), in the presence of 1% EtOH. Inserted is a Lineweaver-Burke plot of 1/V as a function of 1/S.

P:O Ratio

The Pi-stimulated respiration of Pi-starved cells was measured, as described in Methods, over a two minute period following the addition of 100 uM ^{32}Pi. The extent of phosphorylation of ^{32}Pi was assayed as described by Avron (1960).

The assays were carried out at 24° with Pi-starved cells suspended to a concentration of one g wet weight cells/100 ml buffer in 50 mM triethanolamine·HCl, pH 7.8, 10 mM KCl and 2 mM $MgCl_2$.

It was found that during the first two minutes after Pi addition 65.6 nmoles Pi/ml were incorporated into a trichloroacetic acid-insoluble fraction; 0.9 nmoles Pi/ml were found to be trichloroacetic acid-soluble phosphate esters. Thus, out of the 100 nmoles ^{32}Pi/ml added, 66.5 underwent phosphorylation and 97% of this phosphorylated label was incorporated into trichloroacetic acid-insoluble polymers, during the initial two minutes. During this time, it was found that 66 nmoles O/ml were consumed. Thus the P:O ratio was calculated to be 1.0

Potassium Ion Flux During Pi Transport

Uptake of Pi in M. lysodeikticus cells is stimulated by K^+ concentrations of up to 100 mM (Friedberg, 1975). Valinomycin (a ionophore specific for K^+) inhibits Pi uptake in high external K^+ concentrations. In external concentrations of 1 mM K^+, valinomycin slightly stimulates Pi transport (Friedberg, 1975).

In this study, the potassium ion flux during Pi uptake was assayed as described in Methods under various conditions. The major limitation of the method is the low sensitivity of the electrode in the presence of high K^+ concentrations. Thus, measurements were made with cells washed and resuspended in buffer containing 1 mM K^+. No K^+ flux is observed in these preequilibrated suspensions in the absence of external additions.

Addition of Pi to Pi-starved cells was accompanied by an influx of K^+. When 0.67 mM Pi was added, K^+ disappeared from the medium an initial rate of 44 nmoles/min/ml (Fig 8). When Pi was added to cells which were grown in the presence of Pi no flux was observed. In addition, it was found that in the absence of sufficient aeration no K^+ flux was observed upon addition of Pi to Pi-starved cells.

Addition of valinomycin , dissolved in ethanol (final concentration of ethanol in reaction mixture was 0.1%) to Pi-starved cells resulted in the disappearance of 320 nmoles K^+/ml from the medium (Fig. 9). After equilibration of K^+ in the presence of valinomycin was achieved, Pi was added, resulting in the disappearance of an additional 380 nmoles K^+/ml (initial influx rate= 128 nmoles K^+/min/ml). Thus, in toto, 70% of the external K^+ vanished from the medium. Addition of 0.1% EtOH as a control resulted in disappearance of 180 nmoles K^+/ml. Upon subsequent addition of Pi, K^+ was taken up at a rate of 34 nmoles/min/ml. Thus, valinomycin stimulated K^+ influx. The addition of Pi resulted in further K^+ accumulation. The initial rate of K^+ accumulation upon Pi addition is increased in the presence of valinomycin. Addition of ethanol in small concentration causes slight K^+ accumulation by the Pi-starved cells.

Cells which were preincubated in buffer for five hours at room temperature lost their capacity to accumulate K^+ in response to Pi addition (Fig. 10). But when valinomycin was subsequently added, the initial rate of K^+ disappearance was 212 nmoles/min/ml and 620 nmoles K^+/ml, or 62% of the external K^+, had been removed from the medium when a steady state concentration was finally attained.

Proton Flux During Pi Transport

The flux of protons upon addition of Pi to Pi-starved cells was assayed as described in Methods. The cells were washed and resuspended in a weak buffer (1mM triethanolamine·HCl, pH 7.2) in order to enable measurement of small changes in pH, and in 1 mM K^+, in order to approximate the conditions to those used for K^+ flux assays. Pi was added as the triethanolammonium salt, to avoid changing the K^+ concentration.

The immediate effect of Pi addition to the cell suspension was a slight change in pH which is due to the difference in pH between the preequilibrated cell suspension and the Pi added, and is reproducible in the absence of bacteria (Fig. 11a). In order not to affect the pH significantly, small Pi concentrations were added.

Addition of 10 uM triethanolammonium phosphate resulted in a net H^+ efflux of 11.1 nmoles/min/ml. Subsequent Pi additions also caused acidification of the medium due to proton extrusion (Fig. 11). Net efflux of protons was observed upon addition of Pi to cells not starved for Pi indicating that the efflux is associated to Pi-starvation.

Upon addition of 11.5 uM fCCP (a proton-conducting ionophore), the pH dropped by 0.07 units in the Pi-starved cell suspension; no subsequent proton efflux was found upon Pi addition. Since proton-conducting ionophores severely inhibit Pi uptake (Friedberg, 1975), this indicates that proton efflux upon addition of Pi to Pi-starved cells is associated with its transport.

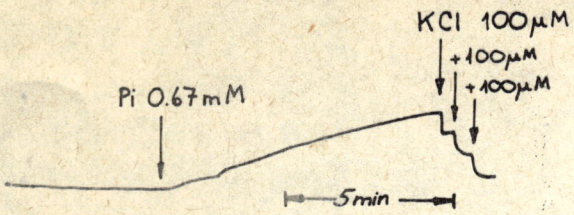

Fig. 8

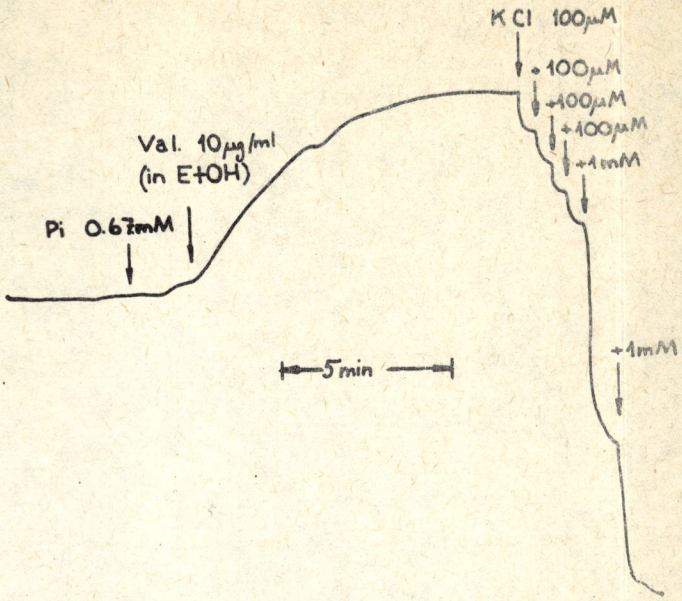

Fig. 10

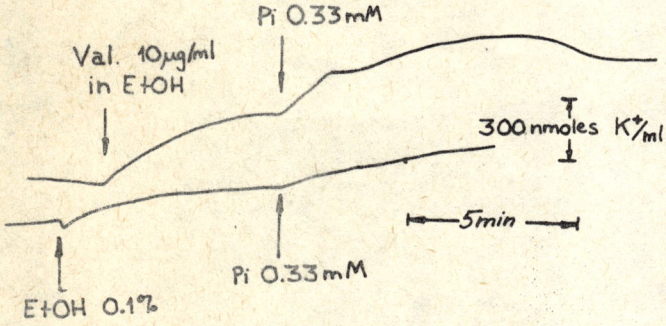

Fig. 9

Fig.8,9,10 - Traces of K^+ flux: Pi-starved cells, washed and resuspended to a final concentration of 1.4 mg protein/ml in 50 mM triethanolamine·HCl, pH 7.8, 2 mM $MgCl_2$, and 1 mM KCl were assayed for potassium ion flux as described in Methods. Pi was added as the triethanolammonium salt. Note that the ordinate axis, representing potassium ion concentration, is logarithmic.

Fig. 11

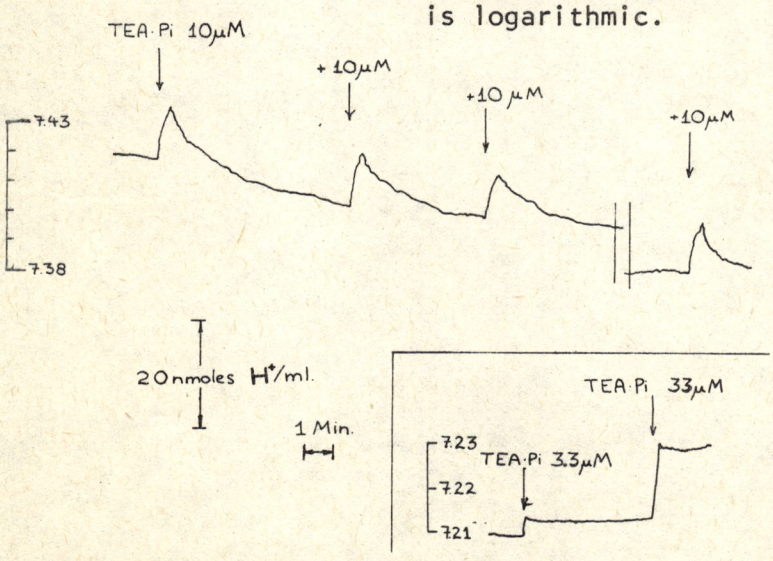

Fig. 11 a

Fig. 11, 11 a - Traces of H^+ flux: Pi-starved cells were washed and resuspended to a final concentration of 1.8 mg protein/ml in 1 mM triethanolamine·HCl, pH 7.6, 2 mM $MgCl_2$, and 1 mM KCl. Changes in pH were monitored as described in Methods. Pi was added as the triethanolammonium salt. Fig. 11 a shows that addition of Pi to buffer causes the initial change in pH in the absence of bacteria; the addition of Pi thus merely slightly changes the pH.

Effect of Magnesium and Calcium Ions on Pi Transport

Mg^{++} concentrations of up to 2 mM stimulate Pi transport in M. lysodeikticus (Friedberg, 1975). It was of interest to see if this was a specific effect or could be duplicated by Ca^{++}, a similar cation.

In Table VII it is shown that in the absence of Mg^{++}, Ca^{++} concentrations of up to 20 mM enhance Pi transport. However, when 2 mM Mg^{++} is present, raising the Ca^{++} concentration increasingly inhibits the transport rate. Thus the stimulation of transport is greater in the presence of Mg^{++} than in the presence of Ca^{++}; Ca^{++} may compete with Mg^{++} competitively for certain sites which have a bearing on the Pi transport rate.

Table VII- Effect of Mg^{++} and Ca^{++} on Pi Transport.

Mg^{++} conc. (mM)	Ca^{++} conc. (mM)	Pi Uptake Rate nmoles Pi/min/mg protein	Relative Pi Uptake (%)
0	0	30.5	100
0	5	32.0	105
0	10	32.0	105
0	20	34.0	111
2	0	39.5	130
2	5	37.5	123
2	10	34.0	111
2	20	32.5	106

Table VII - Pi-starved cells were washed and resuspended in 50 mM triethanolamine·HCl buffer, pH 7.8, 100 mM KCl. Various concentrations of $MgSO_4^*$ and $CaCl_2$ as indicated in the table were added five min prior to ^{32}Pi addition. Pi uptake was assayed as described in Methods.

*The possible effect of the sulfate ion on transport of Pi was also checked; no difference in uptake rate was found.

Uptake of Pi by Isolated Membrane Vesicles

Membrane vesicle preparations, isolated from Pi-starved cells according to the first procedure (see Methods, Membrane preparation, Procedure I) were assayed for Pi uptake, under various conditions. It was found that addition of 0.5 M LiCl to the washing solution, as suggested by Kaback (1971) had a positive effect on retention of Pi by the vesicles. Thus in all Pi uptake assays with vesicles, the membranes on the filter were washed with 5 ml of buffer used in the assay containing, in addition, 0.5 M LiCl.

The rate of uptake in vesicles prepared according to the first procedure was found to be relatively low, and in several reaction mixtures some of the ^{32}Pi initially taken up by the vesicles in the first minute of transport was lost. The initial uptake rate and maximal uptake level attained within the two minute uptake assay with various electron donors is presented in Table VIII. Succinate and ascorbate-PMS were found to be the best energy sources for transport; transport on ascorbate-PMS was inhibited somewhat by CCCP (a proton-conducting ionophore).

Table VIII - The effect of different energy sources on Pi uptake in vesicles isolated according to Procedure I.

Energy Source (20 mM)	Additions	Initial Rate of Uptake (nmoles Pi /min/mg protein)	Maximal Uptake Level Attained (nmoles Pi/mg protein)
-	-	1.93	0.81
α-glycerophosphate	-	1.45	1.08
β-glycerophosphate	-	1.72	1.39
Succinate	-	3.14	1.48
D,L, lactate	-	2.09	1.22
Ascorbate	PMS (0.1 mM)	4.34	1.48
Ascorbate	PMS (0.1mM); CCCP (10 uM)	2.27	0.95

Table VIII- Membrane vesicles were isolated and assayed for Pi uptake as described in Methods. Membranes were preincubated with energy source and additions for five minutes prior to ^{32}Pi addition. Samples were taken at 15,30,60,120 s. Maximal uptake level attained indicates maximum accumulation within the two minute assay.

Because the uptake observed with the first preparation was low and part of the radioactive label initially accumulated leaked out, the preparation of vesicles and the uptake conditions were modified (see Methods, Membrane Preparation, Procedure II). The dialysis was performed in the cold, in order to inactivate proteolytic enzymes which were reported to be active in lysed preparations of M. lysodeikticus (Gorini and Crevier, 1951). DTT was added to protect sulfhydryl groups, since Friedberg (1975) showed that free -SH groups are necessary for Pi transport in M. lysodeikticus. ATP was employed to stabilize the membranes, a procedure suggested by Y. Avidor (personal communication); a low concentration (0.5 mM) was used to minimize possible Pi contamination by ATP hydrolysis. In the assay for Pi uptake, electron donors were added only 15s prior to initiation of Pi uptake. It has been shown that Pi can be taken up in exchange for dicarboxylic acids (Chappell and Crofts, 1966; Chappell and Haarhoff, 1966; Palmieri et al., 1970; Palmieri et al., 1971; Robinson and Williams, 1970; Hoek et al., 1971). Thus the suspicion existed that long incubation times with dicarboxylic acids might result in uptake of these anions in place of or in exchange for Pi.

In an experiment with vesicles prepared according to the second procedure various energy sources were tested, including NADH, glutamate, α-ketoglutarate, ascorbate-PMS, and succinate. Succinate was found to be the best energy source for transport.

Results of an experiment testing Pi uptake over a twenty minute period are presented in Table IX and Fig. 12. It was found that after an initial uptake of Pi over a two minute period, a slower linear phase of uptake occurs. Initial uptake on succinate was relatively high and was inhibited by CCCP and DCCD; the secondary uptake rate was increased in the the presence of DCCD* and was not appreciably affected by CCCP. The initial uptake rate on glutamate was low.

The effect of respiratory inhibitors on the transport of Pi in vesicles was investigated, using KCN, which is a potent inhibitor of respiration in intact cells (see Results, Tables III and IV). Results are presented in Table X and Fig. 13. The initial uptake rate on succinate was inhibited by 82% in the presence of 1 mM KCN; the level accumulated after six minutes of transport, however, was only slightly lower in the presence of cyanide.

*DCCD may play a role in stabilizing the membrane, perhaps by binding to a membrane protein and preventing leakage of protons (Rosen, 1973). This may explain the higher transport observed in these assays.

Table IX - Pi uptake in membrane vesicles isolated according to Procedure II over a twenty minute period.

Energy Source (20 mM)	Additions	Initial Rate of Uptake	Secondary Uptake Rate
		nmoles Pi/min/mg protein	
Succinate	-	4.40	0.13
Succinate	DCCD (24 uM)	0.80	0.16
Succinate	CCCP (0.1 mM); valinomycin (10 ug/ml)	0.80	0.13
Glutamate	-	0.73	0.13

Table IX - Membranes were isolated as described in Methods (see Methods, Membrane Preparation, Procedure II). Membranes were assayed for Pi uptake as described in Methods, with the following changes: uptake was performed at 30°; eight samples, 100 ul each, were taken at 0.25, 0.5, 1, 2, 5, 10, 20 min. Reaction mixtures were preincubated 3 min with additions and 15 s with energy sources prior to initiation of uptake with ^{32}Pi addition.

Table X - Effect of Cyanide on Pi uptake in membrane vesicles.

Energy Source (20 mM)	Additions	Initial Rate of Uptake nmoles Pi/min/mg protein
Succinate	-	3.2
Succinate	KCN (1 mM)	0.6
Ascorbate	PMS (0.1 mM)	0.6

Table X - Membranes were isolated as described in Methods (see Methods, Membrane Preparation, Procedure II). Membranes were assayed for Pi uptake as described in Methods. Samples were taken at 15s, 30s, 3 min, and 6 min. The assay was performed at 34°. Preincubations with additions five min; 15 s with energy sources.

Although the vesicles prepared according to the second procedure did not show greatly enhanced uptake rates, Pi accumulated was not appreciably lost. The uptake of proline in membrane vesicles prepared according to the second procedure was assayed for comparison (see next section). It was found that proline was taken up by the vesicles.

Proline Uptake in Intact Cells and Isolated Membrane Vesicles

The uptake of proline in whole cells and membranes isolated according to the second procedure (see Methods) was assayed as a control for Pi uptake in the vesicles. It has been shown that the proline transport system in E. coli is firmly embedded in the membrane (Kaback and Statdman, 1966) and is very difficult to solubilize (Kaback and Devel, 1969; Piperno and Oxender, 1966). Because the proline transport system is not easily washed from the membrane proline transport was chosen as a control.

Proline uptake was found both in intact cells grown on P-AVS medium and in membrane vesicles (Table XI; Fig. 14,15).

Table XI - Proline Uptake in Intact Cells

Additions	Initial Uptake Rate (nmoles proline/min/mg protein)	% Inhibition
-	7.7	-
KCN, 1.3 mM	1.8	76.7
KCN, 2.7 mM	1.0	87.0
EtOH, 3%	4.5	41.6
DCCD, 1.5 mM in EtOH, 3%	1.9	75.4

Table XI - Cells grown on P-AVS medium were washed and resuspended in 50 mM triethanolamine·HCl buffer, pH 7.8, 10 mM KCl, and 2 mM $MgSO_4$. Proline uptake was assayed as described in Methods; samples were taken at 15, 45, 60, and 180 s. EtOH and DCCD were added three min prior to initiation of uptake; KCN was added two minutes before uptake.

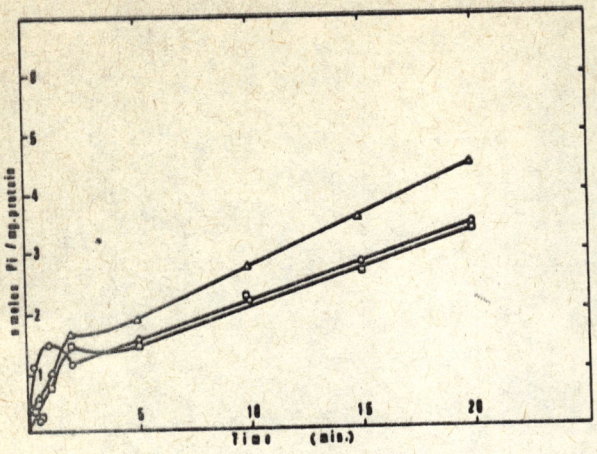

Fig. 12

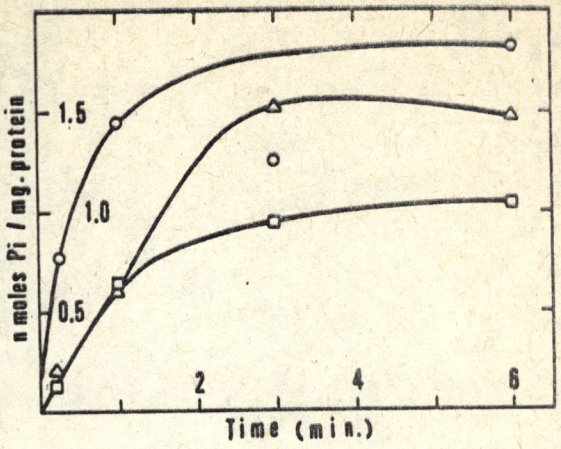

Fig. 13

Fig. 12 - Pi uptake by membrane vesicles. Membrane vesicles were assayed for Pi uptake as described in Table IX. (△), succinate + DCCD; (○) succinate; (□) glutamate.

Fig. 13 - Pi uptake by membrane vesicles. Membrane vesicles were assayed for Pi uptake as described in Table X. Additions: (○), succinate; (△), succinate + KCN; (□), ascorbate + PMS.

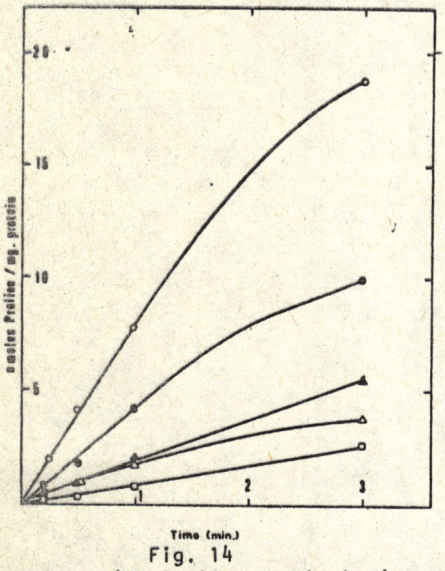

Fig. 14

Fig. 14 - Proline uptake by intact cells was assayed as described in Methods and Table XI. Additions: (○) none; (△),1.3 mM KCN; (□), 2.7 mM KCN; (●),EtOH; (▲),DCCD.

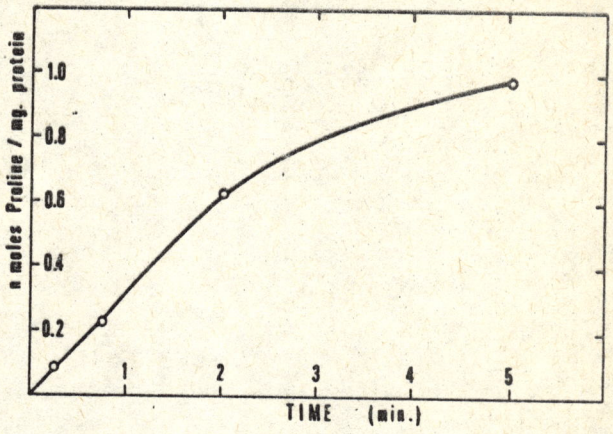

Fig. 15

Fig. 15 - Proline uptake by membrane vesicles. Membranes were isolated from Pi-starved cells according to Procedure II (see Methods). Proline uptake was assayed as described in Methods. 20 mM succinate was added as energy source three min before initiation of uptake. 100 uM ^{14}C-proline was added at zero time. Samples of 200 ul were taken at 15, 45, 120, 300 s.

Intact cells take up proline at an initial rate of 7.7 nmoles/min/mg protein in the absence of an exogenous energy source, under the conditions studied. It was found that ethanol inhibits uptake at a concentration which still stimulates respiration (see Results, Oxygen Consumption, Table III, Expt.V). In E. coli, on the other hand, ethanol has no effect on proline uptake (Berger, 1973). Cyanide is a potent inhibitor of proline transport, as is DCCD (the relatively high DCCD concentration in the experiment presented here permitted a shorter preincubation period; experiments not presented here show that DCCD significantly inhibits proline uptake at concentrations as low as 8 uM).

Vesicles were found to transport proline (Fig. 15), but only the first two minutes of uptake are linear. The initial rate of uptake is 0.32 nmoles/min/mg protein, a value considerably lower than that obtained in intact cells.

Respiration of Isolated Membrane Vesicles

Membranes isolated according to Procedure III (see Methods, Membrane Preparation) respire on a variety of energy substrates (Table XII). Among the energy donors tested, malate was oxidized the most rapidly, in agreement with the results of Grinius et al. (1972). No significant effect was evoked upon addition of Pi or Pi followed by ADP to membrane vesicles respiring on malate. On the other hand, Ishikawa and Lehninger (1962) found that respiration of sonicated membrane vesicles of M. lysodeikticus increased upon addition of Pi alone. The preparation of vesicles studied here respired much slower than the sonicated preparation of Grinius et al. (1972).

The rate of respiration on succinate was found to decrease with time (Table XII). A similar phenomenon was found by Shah and King (1966) (see also Introduction) and may be due to end-product inhibition.

Table XII - Respiration Rate of Isolated Membrane Vesicles on Various Substrates

Assay	Addition	Rate of Oxygen Consumption (nmoles O_2/min /mg protein)	Remarks
a	None	0	
b	Succinate, 10 mM	5.6	Initial Rate after succinate addition
		1.1	Rate 30 min after succinate addition
c	L-lactate, 10 mM	10.9	
d	D-lactate, 10 mM	3.9	
e	β-glycerophosphate, 10 mM	3.4	
f	Malate, 10 mM	40.3	
	Pi, 2 mM	40.3	Added 2 min after malate
	ADP, 1 mM	36.0	Added 2 min after Pi

Table XII - Membranes isolated according to Procedure III (see Methods, Membrane Preparation) were suspended to a concentration of 1 mg protein per ml in 50 mM triethanolamine·HCl, pH 7.8, 10 mM KCl, and 5 mM $MgCl_2$. Oxygen consumption was assayed as described in Methods. Rates are linear with time unless otherwise indicated.

Carotenoids as a Possible Marker for Changes in Membrane Potential

Changes in the absorption spectra of photosynthetic membranes upon illumination is a general phenomenon. Some of these spectral changes, for example the shift at 515-520 nm in higher plant chloroplasts, have been ascribed to changes in light absorption of the carotenoids (Crofts et al., 1971); this shift may also be observed by means other than illumination in which an electric potential across the membrane is perturbed (Jackson and Crofts, 1969). Similarly, the extent of the shift may be attenuated by ionophores (Crofts et al., 1972). It has been postulated that the shift in carotenoid absorption spectrum is an expression of changes in the membrane potential (Witt, 1967; Witt et al., 1967,1968; Fleischman and Clayton, 1968).

Witt et al. (1969) suggested that the pigment shifts in chloroplasts arise from a Stark effect: the electric field across the membrane induces dipoles in the carotenoid molecules and thus causes an alteration in the light absorption pattern.

Upon illumination of photosynthetic bacteria, changes in absorption, characteristic of carotenoid shift, are observed (Smith and Ramirez, 1960; Nishimura and Chance, 1963; Olson and Morita; Vredenberg, Amesz and Duysens, 1965; Amesz and Vredenberg); these shifts are in the range of 450-540 nm.

M. lysodeikticus cells have a variety of carotenoid pigments (Prasad and Litwack, 1961; Gilby and Few, 1958; Rothblatt et al., 1964). We thought that changes in membrane potential caused by either addition of Pi to Pi-starved cells, anaerobiosis, or the addition of valinomycin in the presence of K^+ might induce a shift in the carotenoid spectrum of intact cells.

Because of the problems of light-scattering and sedimentation encountered in spectrophotometric work with intac cells, a cuvette whose geometry was similar to that described by Butler (1972) was employed. Absorption was measured with a Cary XV Spectrophotometer on line to a Hewlett-Packard 2100 Computer. The absorption was measured with an EMI 9558 phototube with S-20 sensitivity. Each spectrum was recorded in the range of 400-500 nm (the three major absorption peaks of the carotenoids of M. lysodeikticus are in this region (Rothblatt et al., 1964)) and recorded in the computer memory. Absolute and difference spectra were reproduced by a strip-chart recorder.

Pi-starved cells were suspended one g wet weight cells per 100 ml buffer composed of 50 mM triethanolamine·HCl, pH 7.8, 10 mM KCl and 5 mM $MgCl_2$. Various concentrations of Pi, valinomycin, succinate and dithionite were added separately and combined in attempts to evoke changes in the membrane potential and thus, perhaps, in the absorption spectrum. The only significant peak in the difference spectra was in the vicinity of 430 nm which was observed upon addition of dithionite or by allowing the cells to run anaerobic. This peak could be partially abolished by reaerating the cells, and probably corresponds to the "Soret Band", indicating cytochrome reduction.

If there is indeed a carotenoid shift under these conditions, the sensitivity of the apparati was not sufficient to measure it. The lack of an observable carotenoid shift may indicate that in M. lysodeikticus the membranal carotenoids are not oriented as in the case of photosynthetic membranes. Thus, carotenoid shift can not be used as an internal probe for measuring changes in membrane potential of M. lysodeikticus under these conditions.

DISCUSSION

Pi Uptake in Isolated Membrane Vesicles

Although membrane preparations isolated from Pi-starved cells do take up Pi, the characteristics of this uptake differ greatly from those exhibited by intact cells.

Perhaps the greates discrepancy between uptake in whole cells as opposed to vesicles is in magnitude. While the Pi-starved intact cells take up 50-100 nmoles Pi/min/mg cell protein under normal conditions, vesicles were found capable of initial uptake rates of up to 5 nmoles Pi/ min/mg membrane protein.

Several explanations for the relatively poor Pi uptake in vesicles can be suggested. The transport of Pi into the cell may be largely dependent on its subsequent metabolism, which is absent in vesicles. Another explanation is that a periplasmic binding protein, specific for Pi, similar to that isolated in E. coli (Medveczky and Rosenberg, 1970), which is necessary for Pi transport is lost during the preparation of the membranes. However, the existence of such a protein in M. lysodeikticus has yet to be demonstrated. An additional possibility is that during membrane preparation there was a large degree of uncoupling between the energy-supplying system and the transport system. Finally, the membranes may have been damaged during preparation by proteolytic enzymes (Gorini and Crevier, 1951) or phospholipases (Kusaka, 1975). On the other hand, the fact that the vesicles were capable of accumulating proline indicates that they were not grossly damaged in preparation.

The uptake of Pi in whole cells is linear over the first twenty minutes (Friedberg, 1975), while in vesicles prepared according to Procedure II (see Methods) two phases of uptake were found: a relatively rapid accumulation over the initial two minutes, followed by a slow linear phase. The initial phase is inhibited by CCCP and DCCD, whereas the second phase is stimulated by DCCD and unaffected by CCCP. The uptake kinetics are similar to those found in E. coli and B. cereus (Rosenberg and La Nauze, 1968; Rosenberg and Medveczky, 1971). In these microorganisms two phases of Pi uptake were also observed - a rapid initial accumulation, followed by a slower subsequent uptake.

Succinate appears to be the best energy source for Pi transport in the vesicles, despite the poor respiration of various membrane prep-

arations on succinate (see Results, Respiration of Vesicles; Grinius et al., 1972; Gorneva and Ryobova, 1974; Shah and King, 1966). This is in accordance with the observation that the substrate most rapidly oxidized (in this case, malate) is not necessarily the best energy source for transport in vesicles (Lombardi et al., 1974).

Reports of Pi uptake in membrane vesicles isolated from bacteria have not been reported in the literature.

Effect of Pi on Oxygen Consumption of Pi-starved Cells

The rate of oxygen consumption by Pi-starved cells is considerably increased upon addition of Pi to the cell suspension. This effect is specific for Pi (or its analog, arsenate) on the one hand, and for Pi-starved cells on the other. Addition of Pi to cells grown in presence of Pi does not influence the respiration rate. Addition of other ions also has little or no effect on the respiration of Pi-starved cells.

A possible explanation for this effect is that it is linked to the transport - Pi stimulates respiration because additional energy is needed for the transport. Experimental results dispell this explanation. The Km for Pi uptake was found to be 4.3 uM (Friedberg, 1975) whereas the external Pi concentration necessary for half-maximal stimulation of respiration is 85 uM. The Pi transport system is thus saturated at low external Pi concentrations while much higher concentrations of external Pi are needed to achieve maximal increase in the respiration rate. In addition, the stimulation of respiration by a given Pi concentration increases considerably when an energy source (succinate or ethanol) is present, whereas Pi uptake is barely affected by the presence of these energy sources (Friedberg, I., personal communication). Thus little correspondence exists between Pi uptake per se and Pi stimulation of respiration.

Another possible explanation is that the effect of Pi on respiration is due to intracellular reactions concurrent with accumulation of Pi within the cell. We showed that Pi added to starved cells is rapidly polymerized and that a correlation exists between the amount of oxygen consumed and amount of Pi esterified and polymerized (see Results, P:O Ratio).

The stimulation in respiration could thus result from respiratory control exerted by the ATPase, or perhaps from the reduction of NAD^+ concurrent with the incorporation of Pi into glyceraldehyde 3-phosphate to form 1,3 diphosphoglyceric acid. Were the stimulation to result from internal reactions of this nature, however, then the stimulation of respiration should reflect the rising Pi concentration in the cell with time during the Pi transport; in other words, the stimulation should be exponential and not linear, unless the Pi concentration within the cell quickly reaches a steady state. Since experimental data indicate that the Pi level in the cell rises continuously during at least the first two minutes of transport, the fact that the respiratory stimulation by Pi is both immediate and linear rule out the role of internal reactions.

One additional explanation is possible - the effect by the Pi is externally-mediated. A site specific for Pi on the membrane is accessible to external Pi, and binding of Pi stimulates respiration. The apparent dissociation constant of the complex-Pi site is 85 uM. Since the stimulation is not observed in cells not starved for Pi this binding site must either be repressed or inhibited when the cells are not Pi-depleted.

Cation Flux During Pi Uptake in M. lysodeikticus

Metabolizing bacteria generally maintain an interior-negative membrane potential, and an internal pH which is alkaline with respect to the medium (Harold, 1972). The active transport of a charged anion, such as phosphate, would require a great expenditure of energy to propel it into the cell against both the electric potential across the membrane and its own concentration gradient (Blum, 1966). In mitochondria it was shown that the transport of Pi is non-electrogenic, meaning that the phosphate enters the cell accompanied by cations which neutralize it (Harold, 1972).

In this work it was shown that under the conditions examined Pi transport in Pi-starved cells of M. lysodeikticus is accompanied by a net influx of K^+ and a net efflux of H^+. Ostensibly, it would appear that Pi transport is coupled to potassium transport. This, however, is not the only possible conclusion. In mitochondria, even though potassium in the presence of valinomycin (a ionophore which catalyzes the electrogenic diffusion of K^+ across the membrane (Harold, 1972)) stimulates transport, experiments have shown that the phosphate is symported with protons and is thus dependent on the proton gradient across the membrane (Chappell and Crofts, 1966; Chappell and Haarhoff, 1967; Mitchell and Moyle, 1969; Palmieri et al., 1970; Coty and Pedersen, 1974).

The proton gradient may be derived from the equation for the proton motive force ΔP (Mitchell, 1973):

$$\Delta P = \Delta\Psi - z\Delta pH \qquad (1)$$

where Δ pH is the proton gradient across the membrane, $\Delta\Psi$ is the electric potential and z is a constant.

Harold (1972) suggests that respiring mitochondria expell protons, thus enhancing the pH gradient. Development of this gradient is limited by the development of the membrane potential (equation (1)); when a

suitable cation can be transported electrogenically into the mitochondrial matrix, protons can then be extruded without disturbing the membrane potential; thus $\Delta\Psi$ can be converted to ΔpH. This can explain why Pi uptake in mitochondria is stimulated in the presence of K^+ and valinomycin. The view that the phosphate transport is a primary anion pump (Pressman, 1970) is contradicted by the electroneutrality of Pi transport in the mitochondria.

Results obtained in our laboratory with M. lysodeikticus can be explained similar to the preceding explanation in mitochondria. In 1 mM external K^+, uptake of Pi (Friedberg, I., personal communication) and K^+ influx during Pi uptake (see Results, Potassium Flux) are stimulated in the presence of valinomycin. Good Pi transport is observed in the absence of external K^+, both in M. lysodeikticus (Friedberg, I., personal communication) and in S. faecalis (Harold and Baarda, 1968), indicating that the potassium is not essential for Pi influx. On the other hand, a correlation was found between starvation for K^+ and Pi uptake in E. coli (Weiden et al., 1967). Their results suggest that Pi transport is dependent on the presence, and perhaps transport of K^+ in this organism.

In experiments conducted with M. lysodeikticus it was shown that proton-conducting ionophores both inhibit Pi uptake (Friedberg, I., personal communication) and abolish proton flux upon addition of Pi to Pi-starved cells (see Results, Proton flux), underlining the importance of the proton gradient for Pi transport.

Our experiments suggest that the proton gradient is necessary for Pi transport in M. lysodeikticus, and although Pi transport is accompanied by considerable K^+ influx, and is enhanced by K^+ concentrations, transport can proceed in the absence of potassium, and is thus not specifically dependent on K^+ influx. The further enhancement of Pi transport in the presence of K^+ + valinomycin is likely due to the ensuing transfer of net positive charge into the cell which enables the extrusion of protons and thus creates a larger proton gradient which in turn drives greater Pi transport. If this is the case, then any cation free to diffuse across the membrane into the cell should have a similar effect. In B. cereus, K^+, Na^+, or triethanolamine stimulate Pi transport (Rosenberg et al., 1969). Similarly, in S. faecalis , K^+, Na^+, NH_4^+ and other cations stimulate Pi transport (Harold and Spitz, 1975). The permeability of these cations

may thus be a factor in their ability to stimulate Pi transport. Similarly, the higher transport observed in M. lysodeikticus in the presence of 50 mM triethanolamine.HCl buffer as opposed to other buffers (Friedberg, I., personal communication) may be due in part to the better permeability of the triethanolammonium cation.

If the uptake of Pi in M. lysodeikticus is accompanied by proton influx, why do we observe net efflux of protons? Perhaps more protons are extruded due to the increase in respiration (see Results, Oxygen Consumption) following Pi addition, than actually accompany the Pi into the cell.

Abbreviations

ATP - Adenosine Triphosphate

ADP - Adenosine Diphosphate

Pi - Orthophosphate

Tris - trihydroxymethyl aminomethane

DTT - dithiothreitol

EtOH - ethanol

CCCP - carbonylcyanide m-chlorophenylhydrazone

fCCP - carbonylcyanide p-trifluoromethoxyphenylhydrazone

PMS - phenazine methosulfate

DCCD - dicyclohexyl carbodiimide

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תאי. אם מוסיפים את הפוספט בנוכחות מקור אנרגיה (כגון אתנול) הקצב המכסימלי של הנשימה ("V max") הוא 30.0 ננמול לדקה למיליגרם חלבון תאי, אך הערך של "Km" נותר ללא שינוי (85 מM). השינוי ב- "V max" מעיד על כך, שיש השפעה הדדית בין הנשימה התלויה במקור אנרגיה לבין הנשימה התלויה בפוספט.

פוספט הנכנס לתאים שהרעבו לפוספט מתפלמר במהירות לפוליפוספט, דרך ATP כתוצר-ביניים. מדידת קצב הנשימה במקביל לקצב סינטזת פוליפוספט מאפשרת את קביעת היחס P:O. נמצא, כי בחידקים מרעבים לפוספט יחד זה שווה ל-1.

טרנספורט של פוספט מלווה בכניסה (נטו) של יוני K^+ אל תוך התאים. נוכחות Valinomycin (שהוא יונופור ספציפי ל- K^+), בריכוזים נמוכים, מזרזת כניסה של יוני K^+. כמו-כן, מלווה טרנספורט של פוספט ביציאה (נטו) של יוני H^+, ויציאה זו מעוכבת על-ידי carbonyl cyanide m-chlorophenyl hydrazone (CCCP, שהוא יונופור של H^+).

יוני Mg^{++} מזרזים במקצת את קצב הטרנספורט של פוספט לחידקים. (הקצב עולה ב-30%, לערך). יוני Ca^{++} אף הם מזרזים את קצב הטרנספורט (בכ-15%). אך אם מוסיפים יוני Ca^{++} בנוכחות יוני Mg^{++}, גורמים יוני ה- Ca^{++} לעיכוב הטרנספורט (בכ-15%). נוכחות יוני סולפט אינה גורמת לשינוי בקצב הטרנספורט של פוספט.

מהירות הטרנספורט של פוספט בממברנות שבודדו מחידקים הנה איטית בהשוואה לקצב הטרנספורט בחידקים שלמים (כ-4 ננמול לדגם למ"ג חלבון בממברנות, לעומת ערך של 100 ויותר בחידקים שלמים). ייתכן, כי בעת הכנת הממברנות השתחררו גורמים הדרושים לטרנספורט מן הממברנות. לבקורת, נבדק טרנספורט של פרולין ונמצא כי הן חידקים שלמים והן ממברנות מבודדות מבצעים טרנספורט אקטיבי של פרולין.

נעשו נסויים לבדיקת השינויים בספקטרום הבליעה של קרוטנים בממברנת החידק כתוצאה מתהליכים הקשורים בטרנספורט האקטיבי. שינויים מסוג זה הובחנו בממברנות בהן מתרחשים תהליכים פוטוסינתחיים, ושינויים אלו מעידים על שינויים בפוטנציאל הממברנה. במקרה של חידקי M. lysodeikticus לא הובחנו שינויים בספקטרום הבליעה של קרוטנים עם הוספת פוספט לחידקים מרעבים לפוספט, וכן לא הובחנו שינויים בספקטרום עם הוספת יונופורים לתרחיף החידקים.

תקציר

בעבודה זו נחקרו השינויים החלים בממברנת התא של חידקי *Micrococcus lysodeikticus* בהשפעת טרנספורט אקטיבי של פוספט. נבדקו שינויים בקצב הנשימה; קינטיקה של שינויים אלו; מעבר יונים דרך הממברנה; השפעת יונים על הטרנספורט; השפעת מקורות אנרגיה; טרנספורט של תרכובות נוספות; שינויים ספקטראליים העשויים ללוות את הטרנספורט.

המחקר בוצע הן בחידקים שלמים והן בממברנות שבודדו מחידקים.

הוספת פוספט לחידקים, שהרעבו לפוספט קודם לכן, גורמת להאצה בקצב הנשימה. הוספת מקורות אנרגיה חיצוניים, (כגון סוקצינט ואתנול) אף היא גורמת להאצת קצב הנשימה. אם מוסיפים מקור אנרגיה ופוספט בזה אחר זה, חלה האצה בקצב הנשימה עם כל הוספה. הפיכת סדר הוספת הריאגנטים גורמת לאפקט דומה. נראה, כי קיימת נשימה החלויה בפוספט, ונשימה החלויה במקורות אנרגיה חיצוניים (כגון סוקצינט, אתנול). ארסנט, שהוא אנלוג של פוספט, גורם להאצה בקצב הנשימה, בדומה לפוספט. הנשימה החלויה בפוספט הנה ספציפית לפוספט (ולארסנט), ומופיעה רק בחידקים מרעבים לפוספט. הבקורות הבאות מבססות ממצא זה: (א) בחידקים שגודלו על פוספט, אין הוספת פוספט (וארסנט) גורמת לשינוי בקצב הנשימה. (ב) מקורות אנרגיה מזרזים את הנשימה בחידקים שגודלו על פוספט. (ג) הוספת פרולין, (הנכנס לתא בתהליך של טרנספורט אקטיבי) אינה גורמת לשינוי בקצב הנשימה, הן בחידקים שגודלו על פוספט והן בחידקים שהרעבו לפוספט. כלומר - טרנספורט אקטיבי כשלעצמו לא גורם לשינוי ניכר בקצב הנשימה. (ד) הוספת מלחים שונים אינה משנה את קצב הנשימה של החידקים. מכאן ששינוי קצב הנשימה אינו נובע משינוי חוזק יוני, והוא ספציפי לפוספט (ולארסנט).

נבדקה הקינטיקה של שינוי בקצב הנשימה כתלות בריכוז פוספט חיצוני, בחידקים מרעבים לפוספט. הקינטיקה שנמצאה הנה מטיפוס קינטיקת Michaelis-Menten. ריכוז הפוספט הגורם למחצית ההאצה המכסימלית בקצב הנשימה (ריכוז אנלוגי ל- Km) הנו 85 µM. ערך זה שונה מערך Km של טרנספורט אקטיבי של פוספט בחידקים, שהוא 4.3 µM. הקצב המכסימלי של הנשימה (אנלוגי ל- V max) כתוצאה מהוספת פוספט הוא 15.6 ננמול לדקה למיליגרם חלבון

אוניברסיטת תל-אביב

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